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Anti-Mi2 autoantibodies target the PHD fingers of SP140L and TIF1y, while anti-TIF1y autoantibodies primarily recognize epitopes outside the PHD region of TIF1y.

Iago Pinal-Fernandez^{1, 2,*}, Jon Musai^{1,*}, Maria Casal-Dominguez^{1, 2}, Katherine Pak¹, Mariana Kaplan³, Blake M. Warner⁴, Lisa G. Rider⁵, Rohit Aggarwal⁶, Chester V. Oddis⁶, Siamak Moghadam-Kia⁶, Gloria Garrabou^{7,8,9}, Albert Selva-O'Callaghan^{10,11}, Jose C. Milisenda^{7,8,9}, John A., Chiorini¹², Andrew L. Mammen^{1, 2,#}, and Peter D. Burbelo^{12,#}

**# These authors contributed equally to this project.*

¹Muscle Disease Section, National Institute of Arthritis and Musculoskeletal and Skin Disease, National Institutes of Health, Bethesda, MD, USA.

²Johns Hopkins University School of Medicine, Baltimore, MD, USA.

³Systemic Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, USA.

⁴Salivary Disorders Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA.

⁵Environmental Autoimmunity Group, Clinical Research Branch, National Institute of Environmental Health Sciences, National Institutes of Health, Bethesda, MD, USA.

⁶Division of Rheumatology, University of Pittsburgh, Pittsburgh, PA.

⁷Muscle Research Unit, Internal Medicine Service, Hospital Clinic, Barcelona, Spain.

⁸Barcelona University, Barcelona, Spain.

⁹CIBERER and IDIBAPS, Barcelona, Spain.

¹⁰Systemic Autoimmune Disease Unit, Vall d'Hebron Institute of Research, Barcelona, Spain.

¹¹Autonomous University of Barcelona, Barcelona, Spain.

¹²Adeno-Associated Virus Biology Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA.

Address correspondence to: Dr. Peter D. Burbelo, National Institute of Dental and Craniofacial Research, National Institutes of Health, 10 Center Dr., Bldg. 10, Rm. 1A01, Bethesda, MD 20892. E-mail: burbelop@nidcr.nih.gov. Andrew L. Mammen M.D., Ph.D., or Iago Pinal-Fernandez M.D., Ph.D., Ph.D., Muscle Disease Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, 50 South Drive, Room 1141, Building 50, MSC 8024, Bethesda, MD 20892. E-mail: andrew.mammen@nih.gov, iago.pinalfernandez@nih.gov

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ABSTRACT

Objectives: Plant homeodomain (PHD) fingers are present in many chromatin-binding proteins. We recently discovered that anti-Mi2 autoantibodies recognize PHD fingers in Mi2 and AIRE. The purpose of this study was to characterize anti-Mi2 autoantibody recognition of PHD fingers in SP140L and TIF1 γ as well as to explore recognition of TIF1 γ by both anti-TIF1 γ and anti-Mi2 autoantibodies.

Methods: Luciferase immunoprecipitation system (LIPS) assays were performed to detect autoantibodies against full-length and protein fragments of SP140L and TIF1 γ in serum samples from myositis patients, disease controls, and healthy controls.

Results: Anti-Mi2 autoantibodies recognized SP140L. When a 49 amino acid fragment of the PHD finger of SP140L was used as the target, the specificity for selectively detecting anti-Mi2 autoantibodies increased. Additionally, anti-Mi2 autoantibodies weakly bound TIF1 γ compared to anti-TIF1 γ autoantibodies. Excluding the TIF1 γ PHD finger from the TIF1 γ target autoantigen eliminated cross-reactivity with anti-Mi2 autoantibodies, confirming that anti-Mi2 autoantibodies specifically target the PHD finger of TIF1 γ . Switching two amino acids in the TIF1 γ PHD finger to resemble those in AIRE markedly enhanced anti-Mi2 autoantibody immunoreactivity. Anti-TIF1 γ autoantibodies primarily recognized the N-terminal fragment outside of the PHD finger, indicating this region contains the immunodominant epitopes.

Conclusions: Anti-Mi2 autoantibodies recognize the PHD fingers of SP140L and TIF1 γ . TIF1 γ is recognized by two different myositis-specific autoantibodies: anti-Mi2 autoantibodies bind the C-terminal PHD domain and anti-TIF1 γ autoantibodies predominantly bind the N-terminal region. Removing the PHD finger from the anti-TIF1 γ

target autoantigen can improve the specificity of anti-TIF1 γ autoantibody assays by reducing cross-reactivity with anti-Mi2 autoantibodies.

INTRODUCTION

Myositis is a heterogeneous family of autoimmune diseases that includes dermatomyositis (DM), the antisynthetase syndrome (ASyS), immune-mediated necrotizing myopathy (IMNM), and inclusion body myositis (IBM).(1, 2) Most DM patients have a single myositis-specific autoantibody (MSA) targeting an intracellular autoantigen. The most prevalent MSAs in DM are anti-Mi2,(3) anti-NXP2,(4) anti-TIF1 γ ,(5) and anti-MDA5.(6) Importantly, each MSA is associated with a unique disease phenotype. For instance, adult patients with anti-TIF1 γ autoantibodies exhibit a significantly heightened risk of malignancy compared to other DM patients but have a low propensity for developing interstitial lung disease (ILD).(7) Conversely, those with MDA5 autoantibodies frequently have ILD but only rarely have a coexisting cancer.(3) While each DM patient is thought to have only a single MSA, anti-Mi2 autoantibodies have shown low-level binding to TIF1 γ , posing challenges for the development of a specific immunologic assay for anti-TIF1 γ autoantibodies.(8)

We recently demonstrated that anti-Mi2 autoantibodies contribute to DM disease pathogenesis by accumulating in the nuclei of muscle cells where they disrupt the normal ability of the Mi2-containing nucleosome remodeling and deacetylation (NuRD) complex to repress gene expression. As a consequence, muscle tissue from anti-Mi2-positive DM patients has a unique transcriptomic profile characterized by the aberrant expression of genes not normally expressed in muscle cells.(9-11) We subsequently showed that anti-Mi2 autoantibodies bind to the plant homeodomain (PHD) finger of Mi2.(12) These 50-80 amino acid regions are found in many chromatin-binding proteins

and mediate their interaction with histones, thereby allowing them to regulate gene expression.(13, 14) Taken together, these observations suggest that anti-Mi2 autoantibodies cause the derepression of gene expression in DM muscle by binding to the PHD finger of Mi2 and interfering with the ability of the Mi2/NuRD complex to bind chromatin.

PHD fingers are found in more than 70 proteins, including autoimmune regulator (AIRE) protein(15), which we previously demonstrated is a target of anti-Mi2 autoantibodies.(12) Intriguingly, the TIF1 γ protein also includes a PHD finger, suggesting that anti-Mi2 autoantibodies might cross-react with TIF1 γ due to their recognition of this domain.

In this study, we aimed to explore the breadth of anti-Mi2 immunoreactivity against other PHD finger-containing proteins, including TIF1 γ and SP140L. We also sought to clarify the dual targeting of TIF1 γ by both anti-TIF1 γ and anti-Mi2 autoantibodies.

METHODS

Patients and Serum Samples

Two different cohorts were used to study autoantibodies. The first cohort, comprising 184 patients and controls (myositis, disease controls, and healthy controls), was designed to test and validate whether the PHD domain of SP140L is specifically recognized by anti-Mi2 autoantibodies. The second cohort, including 134 samples from healthy controls, anti-Mi2, and anti-TIF1 γ patients, was used to investigate the immunodominant epitopes of anti-Mi2 and anti-TIF1 γ in the TIF1 γ protein (Supplementary Table 1). The healthy controls and anti-Mi2 patients included in both cohorts were the same.

Myositis serum samples were obtained from patients who either fulfilled Llyod's criteria for inclusion body myositis (IBM) (16) or the Casal and Pinal criteria for other types of autoantibody-positive myositis (1). Patients were classified as autoantibody-positive if they tested positive for autoantibodies against Mi2, TIF1 γ , NXP2, MDA5, Jo1, HMGCR, or SRP using at least one of the following immunological methods: LIPS, ELISA, *in vitro* transcription and translation followed by immunoprecipitation, line blotting (EUROLINE Autoimmune Inflammatory Myopathies 16 Ag (IgG) test kit), or immunoprecipitation from ³⁵S-methionine-labeled cell lysates.

Standard Protocol Approvals and Patient Consents

Serum samples were collected from patients enrolled in institutional review board (IRB)-approved cohorts at the National Institutes of Health (NIH) in Bethesda, MD, the

University of Pittsburgh, PA, and the Clinic and Vall d'Hebron Hospitals in Barcelona, Spain.

The respective studies received IRB approval from the NIH, the University of Pittsburgh, and the Clinic and Vall d'Hebron Hospitals. Written informed consent was obtained from all participants prior to sample collection and study enrollment.

Anti-AIRE and anti-Mi2 α LIPS

Luciferase immunoprecipitation system (LIPS) immunoassays were conducted as previously described.^(17–20) cDNAs encoding proteins and protein fragments of interest were cloned in the luciferase expression vector pREN2. The cDNAs for Sp140L and TIF-1g used for generating these plasmids were derived from synthetic DNA obtained from Twist Biosciences (Supplementary Table 2) and included *Bam*H1 and *Xho*1 restriction sites for directional cloning into the pREN2 eukaryotic expression vector as C-terminal *Renilla* luciferase fusion proteins. All constructs were sequenced to confirm their integrity.

The plasmid expression vectors were then transfected into Cos1 cells using Lipofectamine 3000, and crude lysates were harvested 24–48 hours post-transfection. Following centrifugation of the crude cell lysate, the luciferase activity of the clarified supernatant was determined and then employed as target antigens in immunoprecipitation assays. For these autoantibody measurements, serum samples from myositis patients, healthy controls, and individuals with other autoimmune diseases were diluted in assay buffer A (20 mM Tris, pH 7.5; 150 mM NaCl; 5 mM

MgCl₂; 1% Triton X-100) and 1ml equivalent of each serum sample was incubated in a 96-well microtiter plate for 1 hour along with 10⁴ light units (LU) per well of lysate corresponding to the different luciferase-antigens.. The serum-antigen mixtures were subsequently transferred to microtiter filter plates (Millipore) containing Ultralink protein A/G beads (Invitrogen) and incubated for 1 hour to capture immune complexes. Lastly, the filter plates (Millipore-Sigma) were washed eight times with buffer A and twice with 1× PBS to remove unbound antigens. After the final wash, coelenterazine substrate (Promega) was added to quantify immunoprecipitated Renilla luciferase fusion proteins by measuring light emission (LU) using a Berthold LB 960 Centro microplate luminometer (Berthold Technologies). The cut-off for each construct was defined as the mean value plus five standard deviations of healthy control sera.

Statistical analysis

Dichotomous variables were summarized as percentages and absolute frequencies. Pairwise comparisons of categorical variables between groups were performed using Fisher's exact test. For continuous variables, intergroup comparisons were conducted using Student's t-test.

Sequence homology between protein sequences was analyzed using MUSCLE v3.8 (Multiple Sequence Comparison by Log-Expectation).

All statistical analyses were performed in the R programming language. A two-sided p-value of ≤ 0.05 was considered statistically significant, with no adjustments made for multiple comparisons.

RESULTS

Anti-Mi2 Autoantibodies Recognize the PHD Domain of SP140L

To investigate whether anti-Mi2 autoantibodies also target the PHD fingers of proteins other than Mi2 and AIRE, we identified genes encoding regions of sequence similarity to the PHD finger of Mi2 (<https://prosite.expasy.org/PS01359>). This analysis identified SP140L and SP140 as potential candidates (Figure 1). Due to the nearly identical protein sequence of this region in SP140L and SP140, we focused on SP140L for further investigation using the LIPS assay.

The LIPS assay was used to test this hypothesis with serum samples from healthy controls, myositis patients, and disease controls. As shown in Figure 2a, the full-length SP140L protein in the LIPS assay was predominantly recognized by sera from anti-Mi2 patients across the studied groups. Nevertheless, a small subset of sera from individuals in the other groups also showed recognition of SP140L at levels comparable to those observed in anti-Mi2 patients.

To determine the contribution of only the PHD zinc finger domain of SP140L to immunoreactivity, we focused on the conserved 49-amino-acid segment of SP140L (Figure 2b). Testing of this small protein fragment demonstrated all but two anti-Mi2 patient sera (92% sensitivity) were recognized, which exceeded the prespecified cutoff ($p=2.2e-16$). In contrast to the results seen with the full-length SP140L target antigen, only one anti-TIF1 γ sample and one systemic sclerosis sample showed

immunoreactivity, resulting in an overall specificity of 99%. These findings highlight that anti-Mi2 autoantibodies specifically recognize the PHD zinc finger domain of SP140L.

Anti-Mi2 Autoantibodies Recognize the PHD Domain of TIF1 γ

Based on bioinformatic analysis showing that TIF1 γ also contains a related PHD zinc finger domain (Figure 1), we studied immunoreactivity against TIF1 γ . Using full-length TIF1 γ protein, anti-Mi2 sera showed significant immunoreactivity with TIF1 γ compared to sera from healthy controls but showed lower levels than with anti-TIF1 γ sera (Figure 3a). As shown in Figure 3a, using the full-length TIF1 γ protein to distinguish between anti-TIF1 γ and anti-Mi2 autoantibodies presents challenges, necessitating a tradeoff between false-negative anti-TIF1 γ results and false-positive anti-Mi2 cases.

Given the preferential recognition of PHD zinc finger domains by anti-Mi2 autoantibodies, we hypothesized that excluding the PHD domain, located in the C-terminal region of TIF1 γ , from TIF1 γ in the LIPS assay could improve discrimination between anti-TIF1 γ and anti-Mi2 autoantibodies. Indeed, a modified assay using amino acids 2-585 from the N-terminus of TIF1 γ (Figure 3b) showed that only one anti-TIF1 γ serum sample was misclassified as seronegative, and one anti-Mi2 serum sample was misclassified as seropositive for anti-TIF1 γ ($p=2.2e-16$). Notably, most anti-Mi2 samples had antibody levels similar to those of the healthy controls.

Overall, the modified immunoassay achieved a sensitivity of 98% and a specificity of 99% for anti-TIF1 γ autoantibodies, significantly improving its ability to exclusively detect TIF1 γ seropositive serum samples.

Additional testing of the C-terminus region (amino acids 586-1127) of the TIF1 γ protein showed variable results with immunoreactivity with both anti-TIF γ anti-Mi2 samples (Figure 3c).

To better understand the varying levels of immunoreactivity of anti-Mi2 autoantibodies against the PHD domains across different target proteins, we analyzed the amino acid similarity in detail and examined the crystal structure of the conserved PHD domain in the proteins studied. This analysis identified two discordant amino acid residues in TIF1 γ (Q894 and N895) compared to AIRE and SP140L which were located at the apex of the PHD loop. To test the significance of these two amino acid changes, we switched these two amino acids (Q894R, N895D) in the TIF1 γ C-terminal protein fragment to match the two residues in AIRE, MI2 and SP140L (Figure 1). This modification resulted in a nearly 10-fold increase in the level of immunoreactivity exclusive to anti-Mi2 samples, without affecting reactivity in anti-TIF1 γ samples ($p=7.4e-13$, Figure 3d and Figure 4). Overall, these results highlight how amino acid differences in the PHD region of these antigens explain the relative levels of immunoreactivity and likely affinity seen against these target proteins.

Anti-TIF1 γ Autoantibodies Primarily Recognize Regions Outside the PHD Domain, Regardless of Cancer Status

Anti-TIF1 γ autoantibodies are strongly associated with cancer and often co-occur with autoantibodies against other proteins such as SP4 and CCAR1 (ref). To further explore immunoreactivity, we used the new assay containing only the N-terminus region of TIF1 γ and the results showed a high levels on immunoreactivity regardless of cancer and non-cancer status of the anti-TIF1 γ seropositive patients (Figure 3b-c). These findings underscore that anti-TIF1 γ autoantibodies predominantly target regions outside the PHD finger, irrespective of cancer status.

DISCUSSION

In this study, we discovered several key features of anti-Mi2 and anti-TIF1 γ autoantibodies. First, we establish that anti-Mi2 autoantibodies bind to the PHD fingers of multiple proteins, including SP140L and TIF1 γ . This highlights their broader reactivity profile beyond Mi2, reinforcing the potential to redefine them as primarily "anti-PHD finger" autoantibodies. Second, we show that assays restricted to the PHD finger can significantly improve the diagnostic accuracy for anti-Mi2 autoantibody detection. Third, we identified TIF1 γ as a target for multiple myositis-specific autoantibodies, with anti-Mi2 autoantibodies recognizing the PHD finger in the C-terminus of TIF1 γ , while anti-TIF1 γ autoantibodies primarily bind to the N-terminal region. Finally, we demonstrate that removing the PHD finger from anti-TIF1 γ assays enhances specificity by eliminating cross-reactivity with anti-Mi2 autoantibodies.

Anti-Mi2 autoantibodies were first identified by Dr. Reichlin and Dr. Mattioli, who discovered them prior to characterizing their first binding autoantigen, naming them based on the initial patient, "Mi2," who exhibited this specificity.⁽²¹⁾ The first proteins known to be targeted by anti-Mi2 autoantibodies were subsequently named Mi2a and Mi2b. Our findings refine this discovery by showing that anti-Mi2 autoantibodies target other proteins that include PHD fingers, including AIRE, SP140L, and TIF1 γ .

The SP140L gene is thought to be a recent evolutionarily member of the SP100 family, formed through DNA rearrangements of the neighboring genes of SP100 and SP140.⁽²²⁾ A small subset of patients with primary biliary cirrhosis have been found to

have autoantibodies against SP140L, which is not unexpected given our findings that the full-length protein is recognized by patients with various autoimmune diseases.(22) In contrast, the isolated PHD finger of SP140L demonstrated specific immunoreactivity in patients with anti-Mi2 autoantibodies. Since SP140 and SP140L differ by only two amino acids in their PHD domains, it is likely that SP140 is also a target of anti-Mi2 autoantibodies.

Our prior research has demonstrated that anti-Mi2 autoantibodies can penetrate muscle cells, accumulate in the nucleus, and disrupt the function of their autoantigens.(9, 11) While the Mi2/NuRD complex was the first identified target affected by the internalization of autoantibodies,(9, 11) it is plausible that the dysfunction of other PHD finger-containing proteins also play a role in disease pathogenesis. Investigating these interactions is inherently complex yet fascinating, as it involves unraveling the potential contribution of several PHD finger-containing proteins.

The initial naming of anti-TIF1 γ autoantibodies was based on their immunoprecipitation patterns (anti-155/140)(5) and later revised to reflect their target protein, TIF1 γ .(23) Our findings challenge the accuracy of this nomenclature, as TIF1 γ is targeted not only by anti-TIF1 γ autoantibodies but also by anti-Mi2 (i.e., anti-PHD finger) autoantibodies. Given TIF1 γ 's role as a suppressor of IFN β 1 expression in the nucleus, and the central importance of IFN β 1 signaling across all DM autoantibody groups,(11, 24) it is intriguing to speculate that anti-PHD finger autoantibody internalization may block TIF1 γ 's inhibitory function, leading to the overexpression of interferon beta. Anti-TIF1 γ

autoantibodies preferentially target the N-terminal region of TIF1 γ , which contains RING and B-box zinc fingers, while anti-Mi2 autoantibodies target the PHD finger. Our study opens the possibility that the overexpression of IFN β 1 in both anti-Mi2-positive and anti-TIF1 γ -positive DM may result from the inhibition of TIF1 γ function through distinct mechanisms: interaction with the RING and/or B-box zinc finger domains in anti-TIF1 γ -positive DM and with the PHD finger in anti-Mi2-positive DM.

Practically, our findings provide a clear path for improving diagnostic accuracy in anti-Mi2 and anti-TIF1 γ autoantibody testing. For anti-Mi2 autoantibodies, assays can be optimized by focusing on the PHD finger, while for anti-TIF1 γ autoantibodies, excluding the PHD finger from test antigens can reduce cross-reactivity. These strategies can enhance the diagnostic performance of the assays, facilitating the accurate differentiation of these autoantibodies in clinical settings.

Despite these advances, several key questions remain unresolved. For instance, a comprehensive understanding of potential differences in anti-TIF1 γ autoantibody binding between cancer and non-cancer patients, particularly within the N-terminal region of TIF1 γ , is still lacking. Additionally, although our focus has been on a subset of PHD finger-containing proteins—Mi2, AIRE, SP140L, and TIF1 γ —many others remain unexplored. Finally, the functional implications of these findings, including the molecular mechanisms underlying disease in DM patients with anti-TIF1 γ autoantibodies and the relationship between autoantibody binding sites and clinical manifestations, require further investigation.

In conclusion, this study enhances our understanding of anti-Mi2 autoantibodies as anti-PHD finger autoantibodies, clarifies the differential recognition of TIF1 γ by anti-Mi2 and anti-TIF1 γ autoantibodies, and provides strategies to improve the diagnostic accuracy for both. These findings advance our knowledge of the molecular mechanisms underlying myositis and pave the way for more precise diagnostic and therapeutic approaches.

FIGURE LEGENDS

Figure 1. Sequence alignment of the PHD finger domains from Mi2, AIRE, SP140L, SP140, and TIF1 γ . The defining motif of the PHD region is highlighted in bold, as detailed below. Starting and ending amino acids are noted at the beginning and end of each sequence. In the modified TIF1 γ PHD domain (PHD_TIF1 γ _SWITCH), the two amino acids altered to resemble the AIRE and SP140L PHD domains are underlined.

Zinc finger PHD-type signature (<https://prosite.expasy.org/PS01359>):

C-x(1,2)-C-x(5,45)-[VMFLWIE]-x-C-x(1,4)-C-x(1,4)-[WYFVQHLL]-H-x(2)-C- x(5,45)-[WFLYI]-x-C-x(2)-C.

PHD1_Mi2 α	(379) QDYCEVCQGGGELTLCDTCPRAYELVCLDPFELDRAPEGKWSCPHCEKE (426)
PHD2_Mi2 α	(456) MEYCRVCKDGGELLCCDAGISSYEIHCLNPFLLPDIPNGEWLCPHCTCP (503)
PHD1_Mi2 β	(370) QDYCEVCQGGGELTLCDTCPRAYEMVCLDPDMEKAPEGKWSCPHCEKE (417)
PHD2_Mi2 β	(449) MEYCRVCKDGGELLCCDTCFSSYEIHCLNPFLLPEIPNGEWLCPHCTCP (496)
PHD1_AIRE	(296) EDECAVCRDGGELTCCDGCPRAFELACLSPFLREIPSGTWKCSBCLQA (343)
PHD_SP140L	(403) LDECEVCRDGGELTCCDTCSSRVFEEDCHIPPV-ESEKTFWNCIFCRMK (449)
PHD_SP140	(690) LDECEVCRDGGELTCCDTCSSRVFEEDCHIPPV-EAERTFWNCIFCRMK (736)
PHD_TIF1 γ	(887) EDWCAVCONGGDLCCCEKCPKVFEELTCHVP'TLLSFPSGDWICTFCRD1 (934)
PHD_TIF1 γ _SWITCH	(887) EDWCAVCRDGGDLCCCEKCPKVFEELTCHVP'TLLSFPSGDWICTFCRD1 (934)

: A AA.:AA::: A: A :A A A : . A A A

Figure 2. Anti-Mi2 Autoantibodies Recognize the PHD Domain of SP140L.

Detection of autoantibodies against the full-length SP140L protein (panel A) and the PHD zinc finger domain of SP140L (panel B) in healthy controls, myositis patients, and individuals with other systemic autoimmune diseases, using the luciferase immunoprecipitation system (LIPS). The dotted line represents the cutoff for anti-AIRE autoantibody positivity, defined as 5 standard deviations above the mean. IBM: inclusion body myositis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; SJD: Sjögren disease.

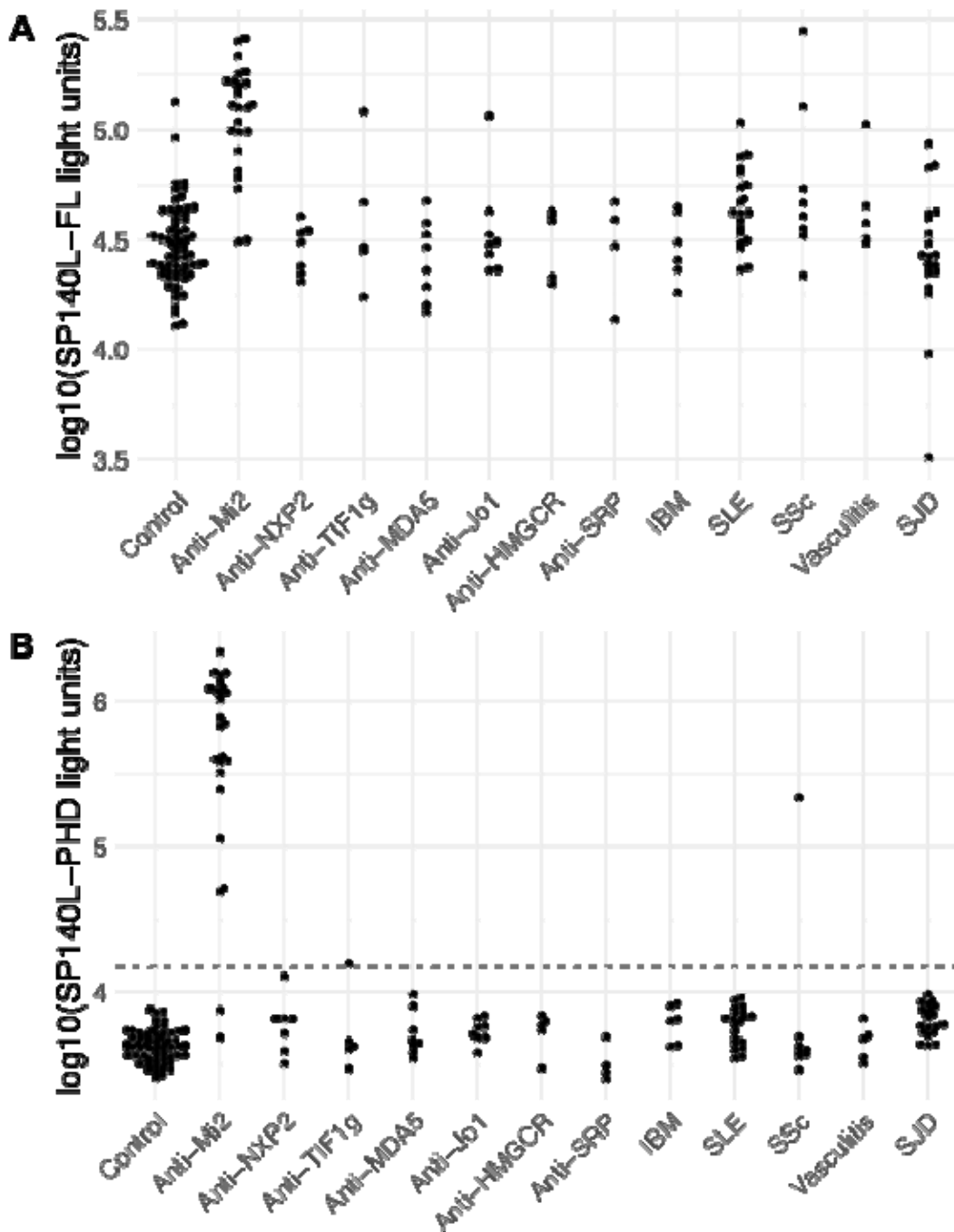


Figure 3. Anti-Mi2 Autoantibodies Recognize the PHD Domain of TIF1 γ and anti-TIF1 γ Autoantibodies Primarily Recognize Regions Outside the PHD Domain.

Detection of autoantibodies was performed using the luciferase immunoprecipitation system (LIPS) against (A) the full-length TIF1 γ protein, (B) the N-terminal region of TIF1 γ , (C) the PHD-containing C-terminal region of TIF1 γ , and (D) a modified C-terminal region of TIF1 γ with two amino acid substitutions to increase similarity to AIRE. Autoantibody levels were analyzed in healthy controls and myositis patients with anti-Mi2 or anti-TIF1 γ autoantibodies. The dotted line represents the cutoff for anti-TIF1 γ autoantibody positivity, defined as 5 standard deviations above the mean. CAM: cancer-associated myositis.

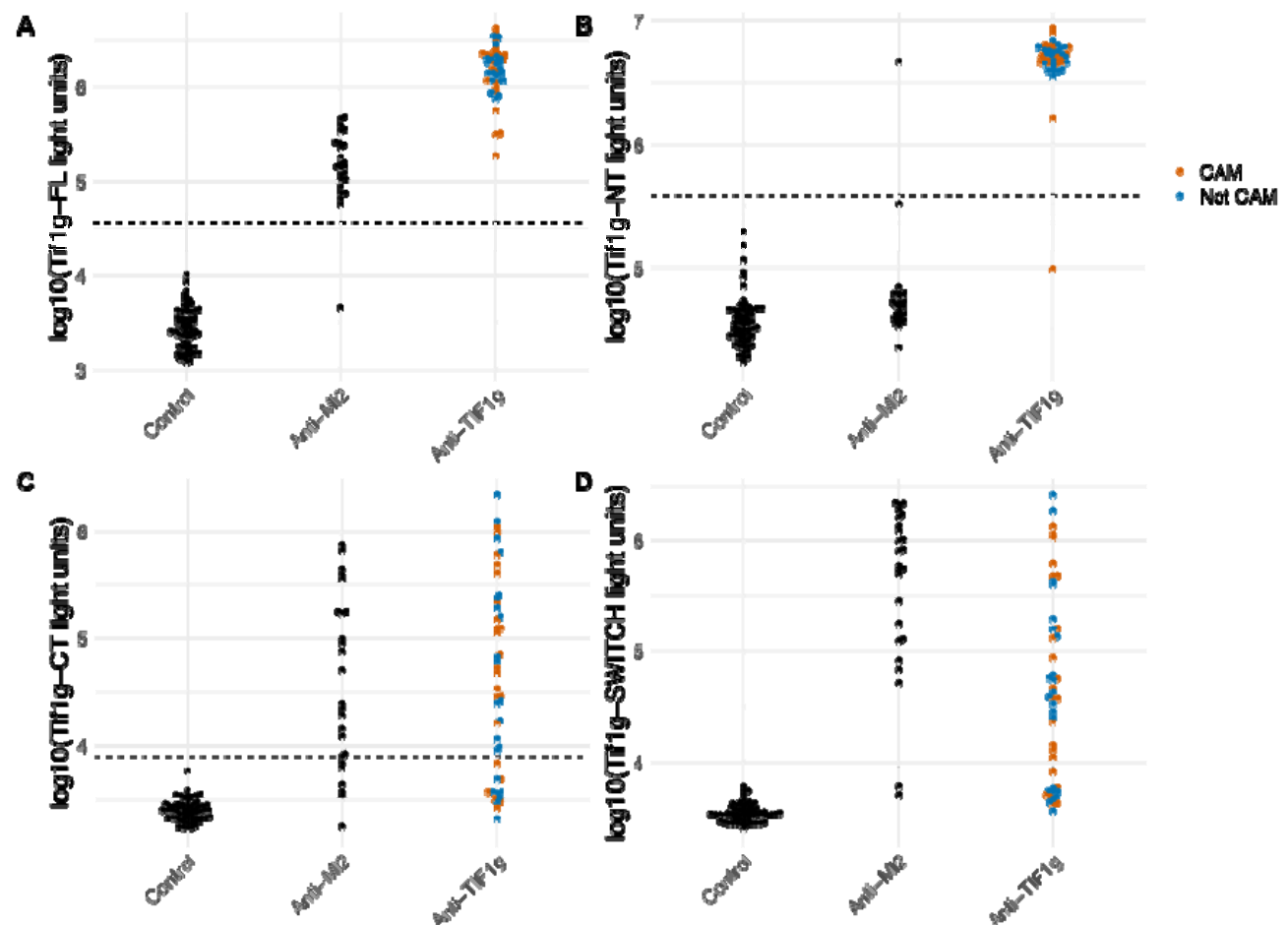
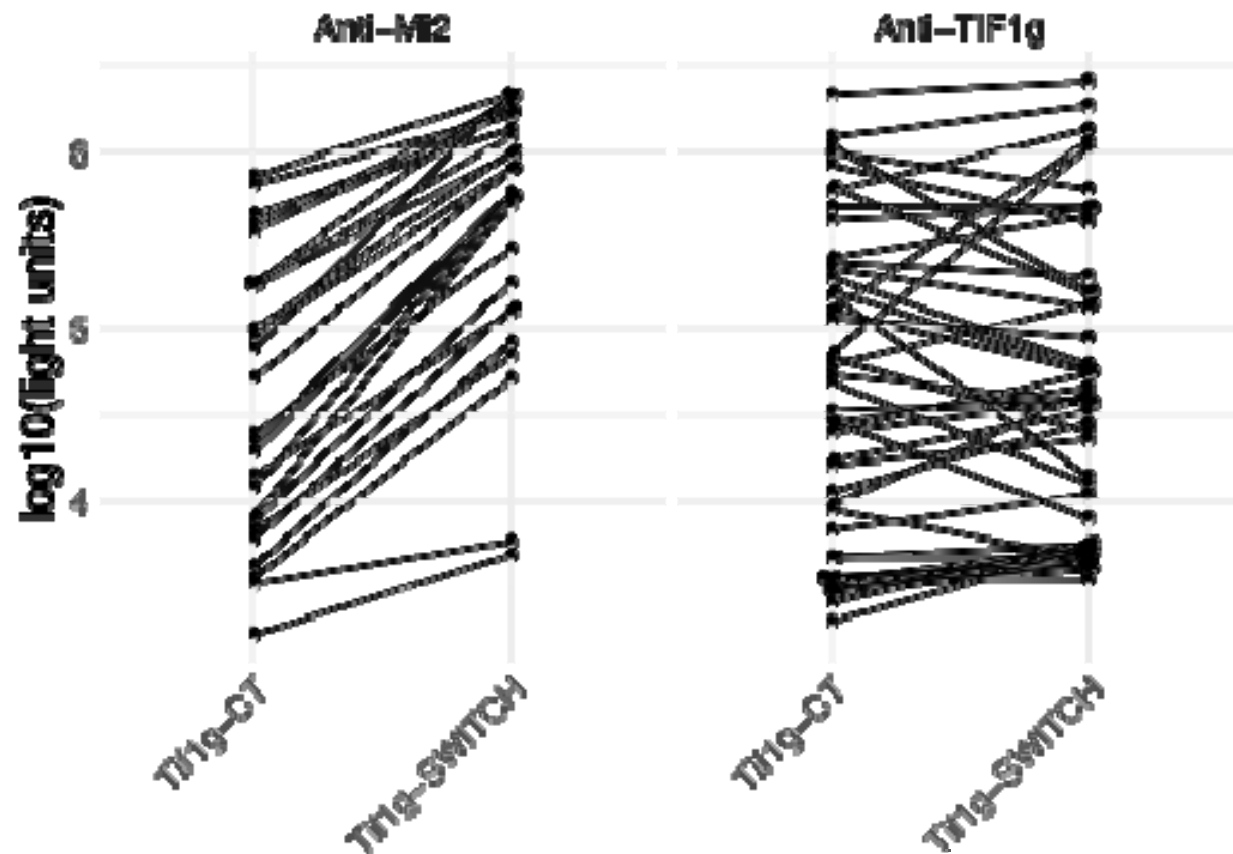


Figure 4. Changing two amino acids in the PHD domain of TIF1 γ increases its immunoreactivity for anti-Mi2 autoantibodies but does not alter its recognition by anti-TIF1 γ autoantibodies. Autoantibody levels were measured using LIPS against the C-terminal region of TIF1 γ (TIF1 γ -CT) or against the same region containing a 2 amino substitution in the PHD domain (TIF1 γ -switch; as described in material and methods) in healthy controls and myositis patients with anti-Mi2 or anti-TIF1 γ autoantibodies.



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